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TRACKING A GENETIC SIGNAL OF EXTINCTION-RECOLONIZATION EVENTS IN A NEOTROPICAL TREE SPECIES: *VOUACAPOUA AMERICANA* AUBLET IN FRENCH GUIANA

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Abstract.—Drier periods from the late Pleistocene and early Holocene have been hypothesized to have caused the disappearance of various rainforest species over large geographical areas in South America and restricted the extant populations to mesic sites. Subsequent improvement in climatic conditions has been associated with recolonization. Changes in population size associated with these extinction-recolonization events should have affected genetic diversity within species. However, these historical hypotheses and their genetic consequences have rarely been tested in South America. Here, we examine the diversity of the chloroplast and nuclear genomes in a Neotropical rainforest tree species, *Vouacapoua americana* (Leguminosae, Caesalpinioideae) in French Guiana. The chloroplast diversity was analyzed using a polymerase chain reaction–restriction fragment length polymorphism method (six pairs of primers) in 29 populations distributed over most of French Guiana, and a subset of 17 populations was also analyzed at nine polymorphic microsatellite loci. To determine whether this species has experienced extinction-recolonization, we sampled populations in areas supposedly not or only slightly affected by climatic changes, where the populations would not have experienced frequent extinction, and in areas that appear to have been recently recolonized. In the putatively recolonized areas, we found patches of several thousands of hectares homogeneous for chloroplast variation that can be interpreted as the effect of recolonization processes from several geographical origins. In addition, we observed that, for both chloroplast and nuclear genomes, the populations in newly recolonized areas exhibited a significantly smaller allelic richness than others. Controlling for geographic distance, we also detected a significant correlation between chloroplast and nuclear population differentiation. This result indicates a cytonuclear disequilibrium that can be interpreted as a historical signal of a genetic divergence between fragmented populations. In conclusion, the spatial genetic structure of contemporary *V. americana* populations shows evidence that this species has experienced large extinction-recolonization events, which were possibly caused by past climatic change.

Key words.—Allelic richness, Caesalpinioideae, chloroplast DNA, climatic changes, cytonuclear disequilibrium, Leguminosae, microsatellite loci, tropical rainforest.

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The long-term dynamics of ecosystems is a crucial factor for understanding species distribution and evolutionary processes (Haffer 1969; Prance 1982; Hewitt 1996; Dynesius and Jansson 2000). The influence of past climatic changes in tropical rainforests, especially in South America, has been largely debated in this perspective (Bush 1994; Colinvaux et al. 1996; Willis and Whittaker 2000). Although the climatic changes during the late Pleistocene (between 22,000 and 10,000 years ago) were weaker in the tropics than at temperate latitudes (Dynesius and Jansson 2000), the geographical distribution of rainforest species might have been seriously affected (Prance 1982; Granville 1988; Rull 1998; Pennington et al. 2000). Based on biogeographical, geomorphological, and paleoecological data, several authors have proposed that the climate was drier and cooler under the tropics during part of the Pleistocene, and that rainforests were therefore fragmented into several isolated geographical areas (the refugia hypothesis: Haffer 1969; Prance 1982; Bush 1994; Hooghiemstra and van der Hammen 1998). The rainforest is assumed to have maintained itself where the environmental conditions (mainly precipitation) were favorable enough, that is, roughly in areas of highest altitude and their vicinity, while lowlands harbored drier forests or savannahs (Hooghiemstra and van der Hammen 1998; Pennington et al. 2000). Several long periods of drought associated with fire events have also been recorded during the Holocene period (between 10,000 ago and now; e.g., Charles-Dominique et al. 1998; Rull 1998; Mayle et al. 2000). These climatic changes may also have caused large extinction-recolonization of rainforest tree populations in lowland areas. Note, however, that some authors analyzing pollen spectra in the Amazon Basin have argued that tropical rainforest distribution did not experience large contractions during the Pleistocene (e.g., Haberle and Maslin 1999; Colinvaux and De Oliveira 2000).

Habitat fragmentation caused by such climatic processes can influence species evolution. For example, it can promote allopatric speciation and contribute to the high species diversity of rainforests (Haffer 1969; Bush 1994; Richardson et al. 2001). It should also have affected genetic diversity within species. As a consequence of founding events and recolonization, populations of species sensitive to climatic

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changes should show less genetic diversity and higher differentiation in areas recently recolonized than in those where these species experienced no (or a reduced) demographic bottleneck in the last generations (Nei et al. 1975; Wade and McCauley 1988; Whitlock and McCauley 1990; Hewitt 1996; Austerlitz et al. 1997). This genetic signal can still be detected ten to hundreds of generations after the last extinction-recolonization event of the species studied (Nei et al. 1975; Austerlitz et al. 1997), depending of course, on the colonization process, on the intensity of gene flow (Wade and McCauley 1988; Wade et al. 1994; Le Corre and Kremer 1998), and on life-history traits (i.e., generation times and population growth rates; see Austerlitz et al. 2000). A gradient of genetic diversity reflecting the occurrence of recolonization at large geographic scale in the Neotropical rainforest during the Pleistocene and Holocene periods (i.e., between 20,000 years ago and now) is especially expected in mature rainforest species, having a low gene flow, a low growth rate, and long generation times. The challenge is to find the convenient genetic markers and species sensitive to climatic change to test for these historical signals.

Cytoplasmic genomes, especially the chloroplast genome in angiosperm species, have been shown to provide convenient markers to trace back recolonization in various organisms (Taberlet et al. 1998; Hewitt 1999). The chloroplast genome is indeed more susceptible to genetic drift due to a smaller effective size and returns more slowly to its initial genetic diversity than the nuclear genome (e.g., Le Corre et al. 1997b; Austerlitz et al. 2000). It is therefore a good marker to trace back seed recolonization from refuge populations (Dumolin-Lapègue et al. 1997a; Taberlet et al. 1998; Hewitt 1999). However, because recombination does not, or rarely, occur (Birky 1995), this genome behaves as a single locus that provides only one realization of processes (e.g., extinction-recolonization, selection, genetic drift) shaping genetic diversity. Therefore, the relative role of these processes cannot be easily disentangled. One solution to trace back evolutionary history of a species is to compare chloroplast DNA (cpDNA) patterns with those derived from several independent nuclear DNA (nDNA) markers. On one hand, isolation during the rainforest contraction and subsequent genetic divergence among separated geographical groups of populations should create (1) similar genetic clustering of populations for the two genomes, provided that pollen and seed gene flow do not differ too much; and (2) cytonuclear disequilibria when comparing nuclear and cytoplasmic allelic frequencies between populations from different geographical origins (Le Corre et al. 1997a). Of course, these genetic signals can be detected only at an appropriate time scale (i.e., around a few tens of generations of the species studied), because nDNA markers are less sensitive to bottleneck and return faster to both the initial genetic diversity and differentiation among populations than cpDNA (see Austerlitz et al. 2000). On the other hand, some nDNA markers (e.g., microsatellite loci) may be extremely polymorphic, presumably due to high mutation rates (Jarne and Lagoda 1996; Goldstein and Schlötterer 1999). This high genetic diversity gives more statistical power when comparing number of alleles within populations between stable areas for species and recently recolonized or disturbed areas (e.g., Spencer et al. 2000).

Patterns of genetic diversity in tree species are useful for retracing the history of ecosystems, because their long generation time slows down the return to demographic and genetic equilibrium after disturbances (Taberlet et al. 1998; Petit et al. 2001). However, most studies have focused on temperate species from Europe, North America, and sometimes from Asia (e.g., Tomaru et al. 1998). Neotropical tree species, especially in South America, have been studied less frequently (but see Aide and Rivera 1998; Caron et al. 2000; Dutech et al. 2000a). These first genetic studies suggested the existence of large-scale recolonization processes that could be explained by past climatic changes in South America. We focus here on the eastern Guiana shield, where it has been suggested that climatic change affected the rainforests during the late Pleistocene (i.e., especially between 22,000 and 10,000 years ago), constraining rainforest species to areas of higher altitude (Granville 1982, 1988). Paleoecological studies based on charcoal analyses showed that the areas of low altitude had also been disturbed during the Holocene (10,000–1000 years ago), as a consequence of natural fire events during the driest periods (Charles-Dominique 1998; Tardy 1998). The definition of those areas having experienced different levels of past disturbance allows us to test genetic consequences of recolonization in Neotropical rainforest species.

The species studied is *Vouacapoua americana* Aublet; a tree restricted to northeastern Brazil, French Guiana, and Surinam. As this species is only present in mature rainforests, has low growth rates (Gourlet-Fleury and Houllier 2000), and should slowly recolonize vacant sites because of limited seed dispersal (Forget 1990; Dutech et al. 2002), climatic change affecting the rainforest species distribution should also have strongly affected its past population dynamics. A preliminary study based on cpDNA markers and sampling covering northern French Guiana, showed the existence of a strong cpDNA geographic structure consistent with recolonization process (Dutech et al. 2000a). In contrast, the spatial genetic structure of nDNA markers (nine microsatellite loci), investigated at the same spatial scale, was not strongly affected by historical factors such as contraction or expansion of populations (Dutech et al. 2004). However, this could result from the fact that nuclear genetic differentiation among recent and old populations decrease rapidly during recolonization, especially in species with long generation times such as trees (Le Corre and Kremer 1998; Austerlitz et al. 2000). Alternatively, recolonization can be detected by the decreasing gradient of genetic diversity expected from old to recent populations (for theoretical examples see Nei et al. 1975; Hewitt 1996; Le Corre and Kremer 1998; Austerlitz et al. 2000; Hewitt 2000).

Gene diversity (H_e; Nei 1987) or allelic richness (A, mean number of alleles per locus) are currently used to compare genetic diversity among populations. Both theoretical and experimental studies indicate that the latter is more informative for detecting recolonization processes than the former (Nei et al. 1975; Spencer et al. 2000; Comps et al. 2001). This difference is caused by the loss of rare alleles after a population bottleneck or during recolonization that affects more strongly and more durably allelic richness than H_e (Nei et al. 1975). Furthermore, refuge populations, which have contributed little to recolonization, are expected to be highly divergent in allelic composition because of past geographical

isolation (Hewitt 1996, 1999). Such a hypothesis can be tested by estimating contribution of populations to allelic divergence (Petit et al. 1998). The goal of this paper is to assess if such a genetic signal can be detected in *V. americana*.

Using 17 and 29 populations for the nDNA and cpDNA analyses, respectively, the following questions are addressed. Does the chloroplast spatial structure differ between areas that are likely to have experienced recolonization versus those supposed not to have been affected by climatic changes during the Pleistocene and Holocene periods (Granville 1982; Tardy 1998)? Can a gradient of allelic richness between these areas and recolonized sites be detected, as expected from theoretical models? Finally, is there a correlation of cpDNA and nDNA allelic frequencies (i.e., cytonuclear disequilibria among populations) that would indicate genetic divergence between isolated geographical areas during the Pleistocene and Holocene periods?

Materials and Methods

Species Studied

Vouacapoua americana (Leguminosae, Caesalpinioideae) is a hermaphroditic, shade tolerant (Gourlet-Fleury and Houllier 2000) tree species of mature tropical rainforests from northern Brazil (Amapá and Pará states), French Guiana, and Surinam (Leite and Lleras 1993). Its local density averages around 10 individuals greater than 10 cm diameter at breast height (dbh) per hectare, but there is large variation in density because of spatial clustering in patches of a few hectares (Forget et al. 1999). The floral syndrome (small, yellow, and fragrant flowers) suggests pollination by small insects (small bees and thrips). Selfing was estimated to occur at a rate of 0.05 (Chauvet 2001). Mature pods generally contain one large seed (mean weight about 30 g; Forget 1990), and fall under the maternal tree. Seeds are dispersed mainly by two rodent species (*Dasyprocta leporina* and *Myoprocta exilis*) that usually bury them less than 10 m from maternal trees, but occasionally up to 30 m (Forget 1990). The occurrence of isolated seedlings suggests that seeds may sometimes be dispersed at greater distance (S. Traissac, pers. comm.).

Sampled Sites

Combining data on the distribution of endemic rainforest species, current levels of precipitation, nature of soil and altitude, and noting the existence of differences between eastern and western floristic affinities in French Guiana, Granville (1982, 1988) inferred the occurrence of four areas where rainforest species were assumed to be always present during the past drier periods (see location in Fig. 1). The regions least disturbed by climatic change would be the central region around Saül and the northeastern area near Cayenne (Montagne Tortue–Kaw). Analyses of the presence/absence of charcoal in soil strongly suggest the absence of important fire events in these regions between 10,000 years ago and now (Tardy 1998). In the two other regions (Montagne Trinité, near the Sinnamary River and Montagne Française-Dékou-Dékou, near Surinam, Fig. 1), the rainforest, although assumed to be always present, probably had a more limited spatial distribution and was more disturbed because of less precipitation (in western French Guiana), lower average altitude, and less hilly areas than in the Saül and Tortue-Kaw areas (for more details, see Granville 1982). A qualitative index (*I*; from one to five) was built on the Granville and Tardy's hypotheses, reflecting the probability of demographic disturbance during the Pleistocene and Holocene periods for each sampled area (Fig. 1, Table 1). The central and northeastern refuge areas were attributed a value of five (very slightly disturbed or not disturbed), and the two other stable areas, the Trinité and Lucifer regions, a value of four (slightly disturbed). Lysis and Nouragues (Table 1) were also given a value of four, because extinction-recolonization of rainforest species during the Holocene (confirmed by charcoal analyses; Tardy 1998) was probably fast, and recolonization occurred from close, undisturbed areas (i.e., Montagne Tortue). The other populations received a value from three to one (moderately, strongly, and very strongly disturbed, respectively), based on their respective distance to the four least disturbed areas and to the coast and the average elevation of the area (Table 1, Fig. 1). All populations close to the coast (Paracou, Counami, and Margot) received an index of one because of the impact of interglacial sea trangression, which occurred around 6000 years ago (Granville 1982; Fig. 1).

Sampling was conducted such as to observe genotypes from different populations of each class of past disturbance (Table 1). Twenty-nine populations were sampled, evenly distributed over the northern half of French Guiana (Fig. 1). In 17 populations, nuclear genetic diversity was estimated using 21 to 24 individuals and five of these individuals, evenly distributed in each population, were analyzed for the chloroplast genome (Table 1). The chloroplast genome was also studied in 12 other populations using two to five individuals such as to improve sampling in northern French Guiana (Table 1). A preliminary study conducted in 14 of these populations showed that a single chloroplast haplotype (chlorotype) occurred in most populations (Dutech et al. 2000a). We chose to increase the number of populations studied for cpDNA diversity, rather than the number of individuals per population, because previous studies showed this strategy to be the most powerful to detect population differentiation (Pons and Petit 1995; Petit and Grivet 2002). Because of limited seed dispersal, individuals sampled for cpDNA were separated by several hundreds of meters in each population to increase the probability of sampling different maternal lineages. The southern part of French Guiana was probably highly disturbed in the past (Granville 1982; Tardy 1998), and should exhibit a lower genetic diversity as compared to the northern part. However, the southern area is hardly accessible, and only a single population was sampled (Fig. 1). In the field, leaves of sampled individuals were stored in plastic bags up to three days after collection and then stored at -80°C in the laboratory.

DNA Analysis

Total DNA was extracted in liquid nitrogen and a CTAB buffer (see Dutech et al. 2000a). The nine microsatellite loci employed as nuclear markers are described in Dutech et al. (2000b). CpDNA was analyzed using the polymerase chain reaction (PCR) and restriction fragment length polymorphism

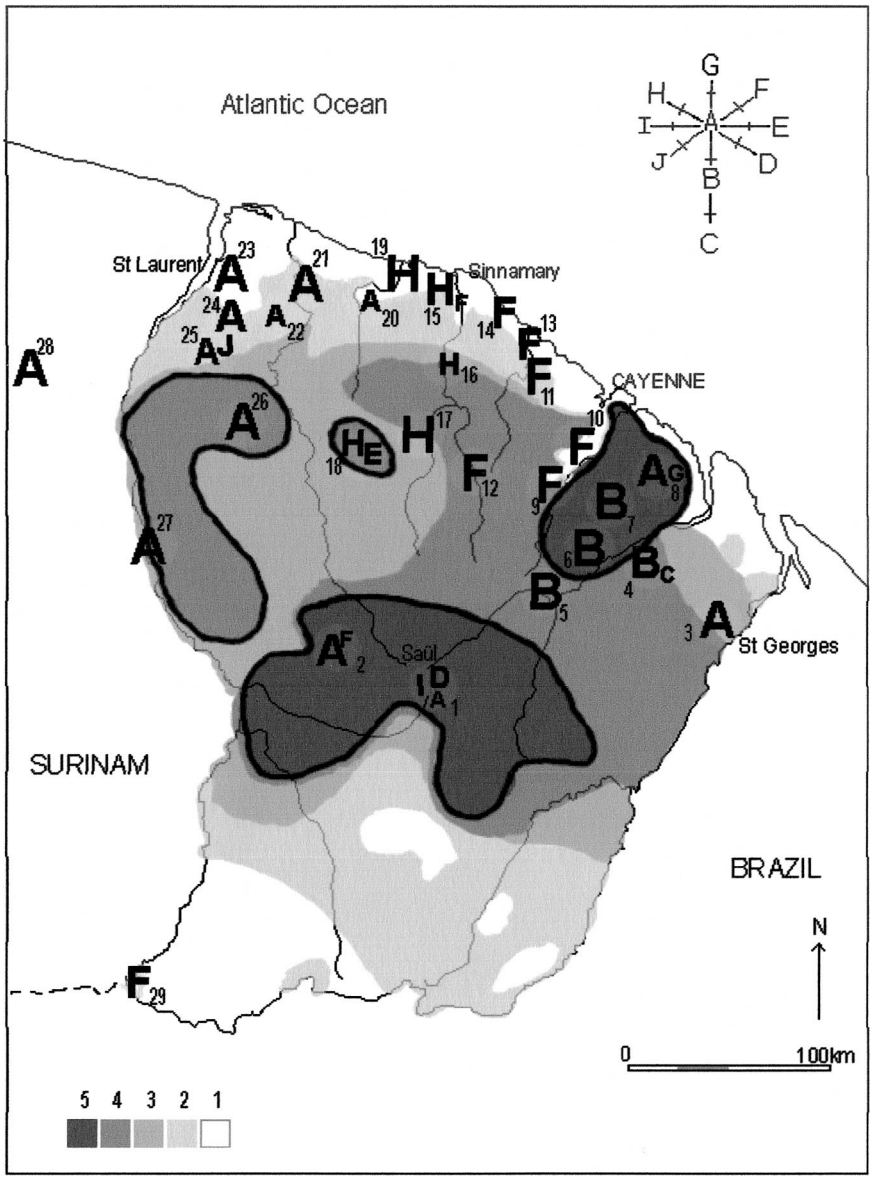

FIG. 1. Geographical distribution of chlorotypes in *Vouacapoua americana* populations from French Guiana. The four areas where rainforest species were assumed to be always present are indicated by dotted lines. The level of assumed past disturbances (*I*; see text) are indicated by different levels of gray (from dark $I = 5$ to white $I = 1$) for each area in French Guiana according to Granville (1982) and Tardy (1998). The genetic relationships among chlorotypes (A to J) are given in the upper right corner. More information about chlorotypes is given in Table 2. The numbers refer to the population numbers given in Table 1. The size of letters is proportional to the number of chlorotypes found within each population.

(RFLP) methods following Dutech et al. (2000a). Two types of mutation are detected by PCR-RFLP, namely point mutation at restriction sites and insertion-deletion (indel) of nucleotides. Both provide appropriate phylogenetic markers if indels are larger than two nucleotides and do not belong to tandem repeats (Gielly and Taberlet 1994). Our preliminary study on *V. americana* cpDNA (Dutech et al. 2000a) suggests that tandem repeats were not included in the amplified fragments. For phylogenetic inference, each variation is considered as one-step mutation, and indel size is not taken into account (e.g., Dumolin-Lapègue et al. 1997a).

Six pairs of primers allowing amplification of intergenic regions from the chloroplast genome were used (Dumolin-Lapègue et al. 1997b), four of which had already been used on *V. americana* cpDNA (Dutech et al. 2000a). The two new primer pairs (CCMP2 and CCMP7) were characterized by Weising and Gardner (1999). The CCMP7 pair amplifies only part of the region originally amplified by the V/L pair (Dutech et al. 2000a), but detected the same length variation in this fragment. However, the V/L pair was removed from the present analysis and replaced by CCMP7 because clearer PCR results were obtained with CCMP7 (C. Dutech, unpubl. data). The PCR conditions for CCMP2 and CCMP7 were identical to those used for the other pairs (Dutech et al. 2000a), except for PCR cycles: the first cycle was 94°C (1 min), 60°C (1 min), and 72°C (45 sec). This was followed by 18 cycles at

TABLE 1. Characteristics of the 29 *Vouacapoua americana* populations studied. Number is the population number in Figure 1. Coordinates refer to the latitude and longitude, respectively. Disturbance is the level of past disturbance experienced during the Pleistocene and Holocene according to the distribution of refuges in French Guiana (Granville 1982; Tardy 1998; see text for additional details). N_{cp} and N_n are the number of individuals sampled for the chloroplast DNA and nuclear DNA analyses, respectively. The approximate length of sampling transects (in km) or areas (in ha) are given. The chlorotypes column gives the names (see Table 2) and number (in parentheses) of chlorotypes found in each population.

Number	Population	Abbreviation	Coordinates	Disturbance	N_{cp}	N_n	Area/transect	Chlorotype
1	Saül	Sau	3°38′N, 5°12′W	5	5	24	100 ha	A(1) D(2) I(2)
2	Dorlin	Dor	3°43′N, 53°32′W	5	5	24	200 ha	A(4) F(1)
3	St Georges	StG	3°55′N, 51°52′W	3	5	24	300 ha	A(5)
4	Virginie	Vir	4°11′N, 52°10′W	4	5	—	5 km	B(4) C(1)
5	Nouragues	Nou	4°05′N, 52°40′W	4	5	21	100 ha	B(5)
6	Tortue	Tor	4°13′N, 52°25′W	5	5	23	350 ha	B(5)
7	Tibourou	Tib	4°26′N, 52°19′W	5	5	24	200 ha	B(5)
8	Kaw	Kaw	4°34′N, 52°14′W	5	5	24	200 ha/9 km	A(4) G(1)
9	Lysis	Lys	4°31′N, 52°31′W	4	5	24	150 ha	F(5)
10	Nancibo	Nan	4°42′N, 52°25′W	1	5	—	5 km	F(5)
11	Balata	Bal	4°57′N, 52°41′W	2	5	24	300 ha	F(5)
12	Saut Takari	STT	4°37′N, 52°56′W	4	5	—	4 km	F(5)
13	Mont. Singes	MtS	5°03′N, 52°42′W	2	4	—	4 km	F(4)
14	Carrière	Car	5°06′N, 52°46′W	1	4	—	4 km	F(4)
15	Paracou	Par	5°18′N, 52°53′W	1	5	24	300 ha	F(1) H(4)
16	Mont. Plomb	MtP	5°01′N, 52°56′W	3	3	—	500 m	H(3)
17	St Eugène	StE	4°51′N, 53°03′W	3	5	24	350 ha	H(5)
18	Trinité	Tri	4°35′N, 53°21′W	4	5	24	100 ha	H(3) E(2)
19	Counami	Cou	5°21′N, 53°12′W	1	5	22	350 ha/8 km	H(5)
20	Organabo	Org	5°20′N, 53°30′W	2	2	—	500 m	A(2)
21	Saut Sabbat	SSa	5°19′N, 53°37′W	2	5	24	300 ha	A(5)
22	Belle Etoile	BEt	5°14′N, 53°40′W	2	2	—	500 m	A(2)
23	Margot	Mar	5°27′N, 54°00′W	1	5	24	200 ha	A(5)
24	Maïpouri	Mai	5°15′N, 53°55′W	2	5	—	2 km	A(5)
25	Voltaire	Vol	5°03′N, 54°05′W	2	5	24	6 km	A(3) J(2)
26	Lucifer	Luc	4°46′N, 53°56′W	4	5	24	200 ha	A(5)
27	Grand Santi	GSa	4°16′N, 54°23′W	3	5	—	4 km	A(5)
28	Suriname	Sur	4°59′N, 55°16′W	unknown	5	—	5 km	A(5)
29	Tri-Jonction	3J	2°25′N, 54°35′W	2	4	—	2 km	F(4)

93°C (45 sec), 59°C with a decrease of 0.5°C per cycle (45 sec) and 72°C (45 sec), and then by 20 cycles at 92°C (30 sec), 50°C (30 sec) and 72°C (45 sec). The final extension was at 72°C for 3 min. The CS and ST pairs, previously used by Dutech et al. (2000a), were not used in the present study because of monomorphism in the first 14 populations studied. The PCR products were digested using restriction enzymes and loaded on acrylamide gels (see Dutech et al. 2000a; Table 2). The short fragments generated with CCMP2 and CCMP7 (about 200 and 500 base pairs, respectively) for which insertion-deletion (indel) events were directly identified on acrylamide gels were not digested.

Data Analysis

The full analysis of genetic diversity, genetic structure within populations, and differentiation among populations at the nine microsatellite loci is extensively presented in Dutech

TABLE 2. Restriction fragments for each of the 10 chlorotypes (A to J) observed in *Vouacapoua americana* for each primer pair. The name of primer pairs (first row) is from Dumolin-Lapègue et al. (1997b), and the restriction enzyme used is given in parentheses. None were used for CCMP7 and CCMP2. The numbers (1 to 3) refer to polymorphic indels (see Dutech et al. 2000a), ranked in order of increasing molecular weight. The approximate difference in base pairs (bp) with the fragment of the A chlorotype is given in parentheses. Number 9 indicates a mutation in a restriction site (RS). *p* is the mean frequency of chlorotypes estimated over the 27 populations in which more than three individuals were sampled.

Primer pairs Chlorotype	HK (*Alu*I)	K1K2 (*Taq*I)	CS (*Hinf* I)	AS (*Hinf* I)	CCMP7	CCMP2	*p*
A	2	1	2	3	2	2	0.348
B	2	9 (RS)	2	3	2	2	0.141
C	2	9 (RS)	2	3	2	3 (10 bp)	0.007
D	2	1	2	3	2	3 (10 bp)	0.015
E	2	1	2	3	2	1 (100 bp)	0.015
F	2	1	2	2 (30 bp)	2	2	0.296
G	2	1	2	1 (50 bp)	2	2	0.007
H	2	1	2	3	1 (20 bp)	2	0.141
I	2	1	1 (10 bp)	3	2	2	0.015
J	1 (10 bp)	1	2	3	2	2	0.015

et al. (2004). Briefly, the main conclusions of these studies were: (1) no departure from Hardy-Weinberg expectations within populations, with the exception of the Nouragues and St Eugène populations, in which a significant heterozygote deficiency was detected; (2) no gametic disequilibrium between pairs of loci; (3) low gene flow estimated among populations; and (4) little evidence of historical effects on the spatial genetic structure. The focus here will be on variation among populations in the mean number of alleles per locus (N_{all}), the observed heterozygosity (H_o), and the gene diversity (H_e; Nei's [1987] method), to test for patterns of extinction-recolonization.

As the presence of rare alleles highly depends on sample size, the mean number of alleles was also estimated using a rarefaction method (El Mousadik and Petit 1996). This parameter ($\hat{r}_n$) is the number of different alleles observed at a given locus when only n genes are considered from the original population sampled:

$$\hat{r}_n = \sum_i \left[1 - \frac{\binom{N - N_i}{n}}{\binom{N}{n}} \right], \quad (1)$$

where N is the total number of genes examined and N_i is the number of occurrences of the ith allele in the sample. $\hat{r}_n$ can directly be compared among populations with unequal sampled size (El Mousadik and Petit 1996). $\hat{r}_n$ was estimated per population and averaged over loci, for a fixed sample size (n) equal to 40, the lowest number of diploid individuals studied being 20 at the Nouragues population for one of the nine loci.

The contribution of the kth population to total allelic richness relative to all other populations ($C_T^R[k]$) was estimated following Petit et al. (1998). The mean allelic richness was computed within populations ($\hat{r}_S[n]$) and over all populations ($\hat{r}_T[n]$). This contribution was computed as:

$$C_T^R(k) = [\hat{r}_T(n) - \hat{r}_{T/k}(n)]/[\hat{r}_T(n) - 1], \quad (2)$$

where $\hat{r}_{T/k}(n)$ is the estimate of allelic richness when the kth population is excluded. $C_T^R(k)$ was also partitioned into its contribution to diversity within populations ($C_S^R[k]$) and to divergence from other populations ($C_D^R[k]$). $C_S^R(k)$ estimates the number of alleles in the kth population relative to the other populations, and $C_D^R(k)$ its allelic differentiation (presence or absence of alleles relative to other populations). The relative contribution to diversity, $C_S^R(k)$, was estimated as:

$$C_S^R(k) = [\hat{r}_S(n) - \hat{r}_{S/k}(n)]/[\hat{r}_T(n) - 1], \quad (3)$$

where $\hat{r}_{S/k}(n)$ estimates the mean allelic richness within populations excluding the kth population. The relative contribution to divergence, $C_D^R(k)$, was obtained by difference between $C_T^R(k)$ and $C_S^R(k)$. When estimating contributions over all loci, the sum of each contribution weighted by its respective $\hat{r}_T(n)$, was divided by the product of the number of loci and the mean $\hat{r}_T(n)$ over all loci minus one. All computations were performed using the program Contrib (available at www.pierroton.inra.fr/genetics/labo/Software). Contributions of populations to gene diversity (H_e) are not presented here, because they do not allow drawing clear predictions when one is interested in extinction-recolonization processes (see introduction). The relationship between historical disturbance and genetic diversity was analyzed by computing Spearman's rank correlation coefficient between the index of disturbance (I; see above) and the mean number of alleles for a fixed sample size ($\hat{r}_{40}$), gene diversity (H_e), and the different contributions to allelic richness ($C_T^R[k]$, $C_S^R[k]$, and $C_D^R[k]$) per population.

The cpDNA analyses were performed using the 27 populations in which more than two individuals were sampled, that is, excluding Organabo and Belle Etoile (Table 1). The mean gene diversity within populations (H_S), total gene diversity (H_T), and genetic differentiation among populations (G_{ST}) were estimated following Pons and Petit (1995) using the computer program Haplodiv (available at www.pierroton.inra.fr/genetics/labo/Software). The standard deviation of these estimates was derived from computations of unbiased variances taking into account the number of populations and individuals sampled (see Pons and Petit 1995). We also estimated F_{ST} using Weir and Cockerham's (1984) method between each pair of populations. The correlation between F_{ST} and geographical distance was tested using a Mantel test. Its significance was assessed through Monte Carlo permutations of the elements of the geographical distance matrix (1000 permutations). F_{ST} was estimated using Genepop version 3.1d (Raymond and Rousset 1995), and Mantel tests were computed using Mathematica (ver. 3.0; Wolfram 1996).

The correlation between nDNA and cpDNA differentiation, independent of the effect of geographical distance, was tested across the 17 populations for which nDNA and cpDNA were studied, using an extension of Mantel test to multiple correlations (Smouse et al. 1986). A partial correlation coefficient can be computed between two matrices for fixed values of a third matrix, which eliminates the effect of the latter matrix on the correlation coefficient. The elements of the cpDNA and nDNA differentiation matrices were the estimates of F_{ST} between pairs of populations. The significance of the partial correlation coefficient between cpDNA and nDNA differentiations was tested by 1000 Monte Carlo permutations of the elements of the nDNA distance matrix, holding cpDNA and geographical distance matrices constant (see Smouse et al. 1986). Under demographic equilibrium, no partial correlation between cpDNA and nDNA differentiation (i.e., no cytonuclear disequilibrium among populations) is expected because of independent evolution of the two genomes. In contrast, contraction and recolonization from different isolated areas should create a correlation, because populations with a common maternal origin (low cpDNA differentiation) should share more nDNA alleles (lower nDNA differentiation), independently of the effect of geographical distance. This result, of course, holds provided that these events are not too old, in which case the nuclear signal would have been eroded (see introduction). The partial correlation between nDNA differentiation and geographical distance, independent of cpDNA differentiation (that is, test for isolation by distance), was tested in the same way.

TABLE 3. Genetic diversity in 17 populations of *Vouacapoua americana* based on nine microsatellite loci. The mean number of alleles (N_{all}), mean number of alleles for a sampling size of 40 genes (R_{40}), observed heterozygosity (H_o), and gene diversity (H_e) are given for each population over all loci. The standard error is given in parentheses.

Population	N_{all}	R_{40}	H_e	H_o
Saül	4.7 (2.9)	3.4 (2.6)	0.501 (0.225)	0.546 (0.275)
Dorlin	5.1 (3.2)	3.8 (2.9)	0.522 (0.242)	0.518 (0.270)
St Georges	4.7 (2.8)	3.3 (2.5)	0.441 (0.215)	0.444 (0.243)
Nouragues	4.0 (2.2)	2.9 (2.1)	0.501 (0.182)	0.473 (0.242)
Tortue	4.8 (2.6)	3.4 (2.5)	0.517 (0.163)	0.532 (0.183)
Tibourou	4.4 (3.5)	3.1 (2.9)	0.437 (0.252)	0.440 (0.244)
Kaw	4.1 (2.7)	2.9 (2.5)	0.484 (0.238)	0.468 (0.249)
Lysis	3.9 (2.0)	2.7 (1.8)	0.508 (0.175)	0.505 (0.196)
Balata	4.2 (2.6)	3.0 (2.4)	0.487 (0.217)	0.509 (0.225)
Paracou	4.1 (2.8)	2.8 (2.5)	0.464 (0.192)	0.417 (0.193)
St Eugène	4.2 (2.3)	3.0 (2.1)	0.460 (0.188)	0.359 (0.215)
Trinité	4.0 (2.8)	2.7 (2.6)	0.464 (0.219)	0.454 (0.242)
Counami	3.7 (1.9)	2.5 (1.8)	0.448 (0.242)	0.441 (0.287)
Saut Sabbat	4.2 (2.3)	3.0 (2.1)	0.506 (0.221)	0.542 (0.241)
Margot	3.6 (1.7)	2.3 (1.4)	0.402 (0.283)	0.338 (0.235)
Voltaire	3.2 (2.1)	2.0 (1.9)	0.340 (0.236)	0.304 (0.202)
Lucifer	3.7 (2.1)	2.5 (2.0)	0.454 (0.243)	0.444 (0.243)

RESULTS

Genetic Diversity

The six pairs of primers used detected six polymorphic fragments in the chloroplast genome (Table 2). The same fragments were observed for those four primer pairs previously used by Dutech et al. (2000a). Most polymorphisms were small indels of a few tens of base pairs, except for a large indel (about 100 bp on the CCMP2 fragment) and a mutation in a restriction site (RS; Table 2). Ten chlorotypes were observed overall, eight of which were separated from chlorotype A by a single indel or a mutated restriction site (Table 2). This produced a simple network of chlorotypes, with chlorotype A being in a central position (Fig. 1). Chlorotype C differed by two assumed mutational steps from chlorotype A (Table 2) and shares one mutation with both chlorotypes B and D. However, B and C share a mutation in a restriction site (K_1K_2 primer pair), while both C and D present a similar indel of 10 bp (CCMP2 primer pair). As different indels may produce the same pattern of migration, it is likely that C and D are different for this fragment and appear identical in size. As a consequence, C was connected to B rather than to D in the chlorotype network (Fig. 1). This assumption could be confirmed by sequencing the fragment generated by the CCMP2 primer pair.

The mean number of alleles (A), number of alleles for a fixed sample size of 40 genes ($\hat{r}_{40}$), observed heterozygosity (H_o), and gene diversity (H_e) over all microsatellite loci are given per population in Table 3. The mean number of alleles per population was 4.1 (maximum = 5.1 in Dorlin; minimum = 3.2 in Voltaire). The mean $\hat{r}_{40}$ was 2.9 with a maximum of 3.8 in Dorlin and a minimum of 2.0 in Voltaire. The mean observed heterozygosity per population and gene diversity were respectively 0.467 and 0.506 (Table 3).

TABLE 4. Spearman rank-order correlation coefficient across populations between the index of past disturbance (I; see text) and several measures of gene diversity, including the mean number of alleles for a sampling size of 40 genes (R_{40}), gene diversity (H_e), and the allelic contribution to total diversity (C_T^R[k]), to diversity within populations (C_S^R[k]) and to divergence from other populations (C_D^R[k]) of the kth population (see text). Asterisks indicate that the coefficient is significantly different from zero at the 0.05(*), 0.01(**), and 0.001(***) level, respectively.

	I	R_{40}	H_e	$C_T^R(k)$	$C_S^R(k)$
R_{40}	0.554*				
H_e	0.428	0.553*			
$C_T^R(k)$	0.779***	0.431	0.564*		
$C_S^R(k)$	0.554*	1.000***	0.553*	0.431	
$C_D^R(k)$	0.376	−0.164	0.271	0.738**	−0.164

Chloroplast DNA Differentiation among Populations and Geographical Location of Chlorotypes

The analysis of cpDNA diversity showed that gene diversity within populations (H_S = 0.13, SE = 0.05) was much lower than total gene diversity (H_T = 0.77, SE = 0.04). Most diversity was distributed among populations (G_{ST} = 0.83, SE = 0.06). The low gene diversity within populations can be explained by the fact that a single chlorotype occurred in 20 populations, and no population had more than three chlorotypes (Fig. 1). Six chlorotypes of 10 were found in one population only at frequencies lower than 0.4. Chlorotype A had the highest frequency over all populations (Table 2) and the widest geographical distribution, being found in all western populations, in the central region, and in the east of French Guiana (Fig. 1). The three other chlorotypes found in several populations (B, F, and H) were mainly located in the central area along the coast (Fig. 1). They occurred as homogeneous patches of several square kilometers roughly delimited by rivers. Rivers are not absolute limits though, because these chlorotypes were often found on both sides of some rivers (e.g., B in population 4 and 6, F in populations 9 and 12; Fig. 1). Chlorotype F was found along the coast and both in one of the two central populations and in the single southern population. The Mantel tests between cpDNA distances and geographical distance over pairs of populations with more than two individuals sampled were not significant ($r < 0.001$, $P = 0.62$).

Relationship between Genetic Diversity and Past Disturbances

The six rare chlorotypes were found only within or very near the four areas assumed the least disturbed in the past, and all frequent chlorotypes (i.e., $p > 0.141$; Table 2) occurred at least in one of these four areas (Fig. 1). The mean number of microsatellite alleles and expected heterozygosity per population were very similar across all populations (Table 3). However, the correlation between I (index of past disturbance) and $\hat{r}_{40}$ was significant at the 0.05 level, but not that with H_e (Table 4). $\hat{r}_{40}$ and H_e were also significantly positively correlated (Table 4). The allelic contributions of single populations to total richness ($C_T^R[k]$), to diversity within populations ($C_S^R[k]$), and to allelic divergence from other populations ($C_D^R[k]$) were low, ranging from −1.5% to 2% (Fig.

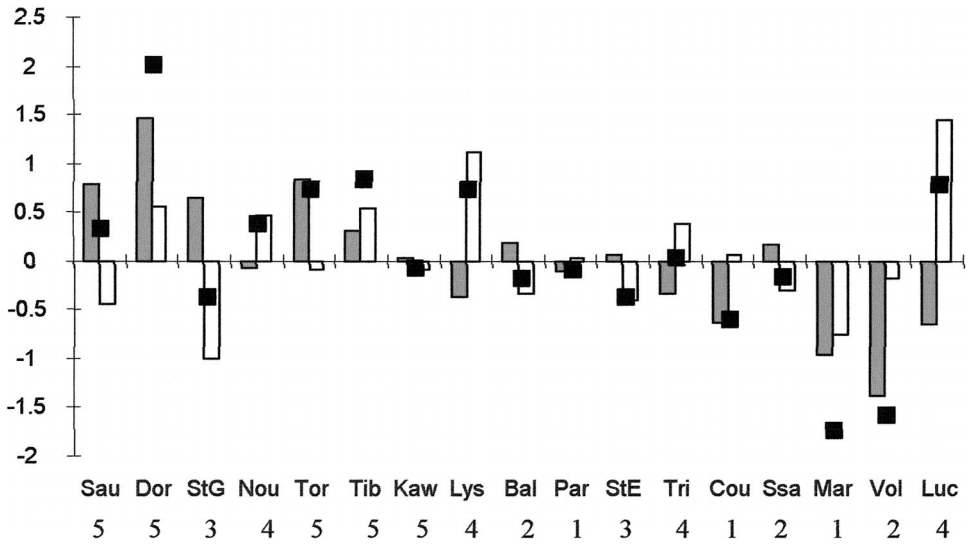

FIG. 2. Contribution (in %) of the *Vouacapoua americana* populations studied to total richness ($C_T^R[k]$ full squares), diversity within populations ($C_S^R[k]$; gray histogram), and divergence from other populations ($C_D^R[k]$; empty histogram), estimated over all microsatellite loci. Abbreviations for population names are given in Table 1. Index of past disturbance (see text for details) is given under abbreviation for each population.

2). All western populations (from St Eugène to Lucifer) had a negative relative contribution to total allelic richness ($C_T^R[k]$), except for the two populations in the assumed slightly disturbed areas (Trinité and Lucifer; Fig. 2). These two populations were characterized by a slightly positive relative contribution to divergence ($C_D^R[k]$). The western populations were also characterized by a lower relative contribution to diversity ($C_S^R[k]$) than the pooled central and eastern populations (Mann-Whitney *U*-test, $U_{7,10} = 61$, $P < 0.01$). $\hat{r}_{40}$ and $C_S^R(k)$ gave the same information (Table 4), which is not surprising because they represent the absolute and relative allelic richness of each population, respectively. Most populations in the four least disturbed areas (six of seven) and their neighboring populations (Nouragues and Lysis) were characterized by a positive relative contribution to total allelic richness (Fig. 2). Both relative contribution to total allelic richness and relative contribution to diversity were significantly correlated with *I* ($r = 0.779$, $P < 0.001$ and $r = 0.554$, $P < 0.05$, respectively), but not relative contribution to total divergence ($C_D^R[k]$) and H_e (Table 4).

Independent of the effect of geographical distance, the cpDNA and nDNA differentiation (F_{ST} estimates) were significantly correlated across pairs of populations ($r = 0.207$, $P = 0.038$). The geographical distance and nDNA genetic differentiation between population pairs were also significantly correlated over all loci ($r = 0.240$, $P = 0.032$), independent of the effect of cpDNA differentiation.

DISCUSSION

Homogeneous Chloroplast Patches in French Guiana

The most striking result of this study is the strong chloroplast structure characterized by large homogeneous patches of a few thousands of hectares exhibiting a single chlorotype and high differentiation among *V. americana* populations ($G_{ST} = 0.83$), confirming preliminary results (Dutech et al. 2000a). Large differentiation among populations is typical of maternally inherited genomes dispersed by seeds, but our result ranks with the highest estimates in tree populations (for estimates of cpDNA differentiation among angiosperm populations, see Raspé et al. 2000). This result is in agreement with a very limited seed dispersal by rodents (mainly up to 30 m; Forget 1990) or suggests a small local effective population size and an important genetic drift. Because the correlation between cpDNA differentiation and spatial distance among the 27 populations studied was nonsignificant and close to zero ($r < 0.001$), however, it is likely that other factors such as extinction-recolonization events also shaped the distribution of chlorotypes in French Guiana.

The distribution of chlorotype A contrasts with the other chlorotypes, because it was found all over the sampled area. Of course the PCR-RFLP technique does not reveal the full variation at the nucleotidic level, and chlorotype A could indeed correspond to several DNA sequences (e.g., in eastern and western populations). This could be evaluated using DNA sequencing. However, an alternative hypothesis is that chlorotype A is the older (ancestral) one, which is suggested by its central position in the chlorotype network (see Templeton et al. 1995). Its extremely large geographic distribution could be explained by an earlier occurrence in French Guiana compared to the other chlorotypes.

Low seed dispersal and recent mutations from an ancestral chlorotype are not sufficient explanations for the occurrence of homogeneous patches of chlorotypes (e.g., F or H), together with the absence of chlorotype A. Although chlorotype A could occur at low frequency in these patches and be undetected, such a situation is unlikely to occur in all homogeneous cpDNA patches sampled on the coast. Selection on cpDNA can also explain this pattern. New chlorotypes could rise in frequency, if they are associated with selectively favored mutations because of intrinsic superiority or environmental change. Because the different chlorotypes observed

in the homogeneous coastal patches (B, F, and H) are not closely related (Table 2, Fig. 1) and because of clonal evolution of the chloroplast genome (see introduction), several advantageous mutations independently occurring in these different areas should therefore be invoked. This assumption is not consistent with low chloroplast mutation rates (Frascaria et al. 1993).

Local extinctions and the recolonization dynamics in *V. americana* may have resulted in the fixation of one chlorotype per population because of cpDNA sensitivity to bottlenecks and drift in small populations (Wade et al. 1994; Austerlitz et al. 2000). Limited seed dispersal in *V. americana* would reinforce this effect. Such an explanation is supported by both demographic (massive mortality documented in several populations) and genetic (detection of bottlenecks) data (Dutech et al. 2004). However, high local extinction rates or extinction on a large spatial scale are probably required to promote the fixation of a unique chlorotype in different areas of several thousands hectares. Such processes might have occurred during the Pleistocene and Holocene climatic changes. We thus expected that more than one chlorotype would be generally detected in the most stable areas for rainforest species defined by Granville (1982) and Tardy (1998) in French Guiana. The occurrence of homogeneous patches of cpDNA along the coast was probably also promoted by rivers, which are strong barriers to *V. americana* seed dispersal. Seeds are too heavy to float in water (S. Chauvet, pers. comm.) and large parts of rivers banks are made of hydromorphic soils that are not favorable for the survival of *V. americana* seedlings (Forget et al. 1999).

Association between Chloroplast DNA and Nuclear DNA Pattern and Large-Scale Extinction-Recolonization in Vouacapoua americana

The similar decrease in allelic richness among *V. americana* populations for both genomes is in agreement with the hypothesis of recolonization of areas supposed to have been highly affected by climatic changes in French Guiana. Furthermore, populations located in areas assumed to have experienced no or limited past disturbance generally exhibited alleles that were not found in areas assumed to have been recently recolonized. The highest contributions of populations to nuclear allelic divergence ($C_D^R[k]$) were indeed generally found in populations with an index of past disturbance equal to four or five (not or weakly disturbed; Fig. 2). Rare chlorotypes were also found within or close to the four putative refuge areas only. Despite an approximate definition of areas characterized by different levels of past disturbances, these empirical results are consistent with the loss of genetic diversity expected from different models of extinction-recolonization over large areas (Austerlitz et al. 1997; Le Corre et al. 1997b; Le Corre and Kremer 1998; Hewitt 2000). A further empirical result supporting the hypothesis of extinction-recolonization processes at large geographical scale is the significant partial correlation of cpDNA and nDNA differentiation. Although this result should be confirmed using populations sampled at larger geographic scale, it strongly suggests an ancient contraction of *V. americana* populations. Our study also showed that both the nuclear and chloroplast allelic richness are significantly higher in the central and eastern populations than in the western ones. This is consistent with the hypothesis that the central and northeastern regions played a primary role in the maintenance of rainforest species in French Guiana during these geological periods (Granville 1982, 1988; Tardy 1998).

The nDNA showed a smaller difference in allelic richness and smaller genetic differentiation among populations than cpDNA (for nDNA differentiation among *V. americana* populations, see also Dutech et al. 2004). Such a difference constitutes a classical result in population genetics that can be explained by differences in the evolutionary forces acting on the two genomes (e.g., ploidy level, mutation and recombination rates, gene flow; see introduction). Strong gene flow mediated by pollen rapidly blurs the nuclear differentiation among populations and may here be invoked (Le Corre and Kremer 1998). However, most of pollen gene flow is limited to a few hundred meters in *V. americana* populations of French Guiana (Chauvet 2001; Dutech et al. 2002). We instead suggest that the observed differences result from different sensitivity to bottlenecks and recolonization events of the two genomes. Both the larger effective population size and the higher mutation and recombination rates of microsatellite loci can explain the limited nuclear signal of recolonization (see Austerlitz et al. 2000). Moreover, even rare events of pollen flow from older toward younger, colonizing tree populations efficiently counteract founder effects in the nuclear genome (Austerlitz et al. 2000).

The only difference in genetic variation among geographical areas was in estimated allelic richness. The absence of correlation between gene diversity (H_e) and the index of past disturbance was expected, because H_e is affected little by population bottlenecks. This confirms previous experimental results (Comps et al. 2001; Spencer et al. 2000). The absence of correlation between the contribution of each population to allelic divergence ($C_D^R[k]$) and the index of past disturbance is more difficult to explain. Populations from stable areas are indeed expected to exhibit more rare alleles, because populations from recolonized areas should especially be affected by sampling effects during recolonization. Although the highest contributions to allelic divergence were only observed in the populations least disturbed in the past, some of these populations exhibited a negative relative contribution to divergence (Saül, Tortue, and Kaw populations; Fig. 2). Several explanations about the absence of positive contribution to divergence for some assumed refuge populations can be given. On one hand, the impact of extinction-colonization (if it occurred) could be weak and sometimes difficult to detect. On the other hand, the contribution to divergence depends on the role of refuge populations in the recolonization process (Hewitt 1996, 1999). Refugia such as Saül, Kaw, and Tortue, could have been sources during *V. americana* expansion and could poorly diverge from recent populations.

Does the Vouacapoua americana Genetic Diversity Reflect Rainforest Contractions during the Pleistocene and Holocene?

The absence or rarity of historical data such as pollen fossil and charcoals strongly limits inferences on the history of

Neotropical rainforests, leading to contradictory arguments on the impact of past climatic change on the vegetation (Colinvaux et al. 1996; Pennington et al. 2000; Willis and Whittaker 2000). One solution has been to infer past vegetation refuges based on current endemism and species distribution. However, more extensive biological collections within the putative refuge areas than in other areas in the Amazonian Basin (Nelson et al. 1990), as well as a larger number of ecological habitats in the refuge areas (Bush 1994), could explain more endemism and species richness. The genetic approach taken in this study offers information that is less susceptible to be biased by these factors. First, assuming neutral evolution of microsatellite nDNA loci and intergenic cpDNA fragments, the genetic diversity is not directly affected by ecological factors, and reflects more specifically the past population dynamics of species (Milligan et al. 1994). Most populations were at Hardy-Weinberg equilibrium at all microsatellite loci (Dutech et al. 2004), and the geographical distribution of cpDNA and nDNA allelic richness was similar, suggesting that selection is not acting on the markers used. Second, as pointed out in the Materials and Methods, the standardization method on allelic richness reduces the bias caused by uneven sampling among populations and presence/absence of rare alleles within the populations sampled. Third, the estimates of allelic contributions only depend on regular sampling among sampled areas (Petit et al. 1998). This prerequisite was fulfilled, since the populations sampled are evenly distributed over the center and north of French Guiana, and areas of various coefficients of past disturbance are equally represented.

This study only suggests differences in past disturbances among geographical areas in northern French Guiana, that is, a small part of *V. americana* distribution area. Whether our results could be extended to the whole distribution area of *V. americana*, that is, to southern French Guiana, Surinam, and especially northern Brazil (Amapá and Pará states), is certainly questionable. However, French Guiana was probably one of the least disturbed areas on the Guiana Plateau (Granville 1982, 1988; Bush 1994). Marked differences in allelic richness should therefore be observed among *V. americana* populations in northern Brazil (Granville 1988; Bush 1994). Furthermore, the large dry area around the Tumuc-Humac Mountains, delimiting the boarder between French Guiana, Surinam, and Brazil, was probably hostile for rainforest species such as *V. americana* during most of the Pleistocene and Holocene periods (Granville 1982). If recolonization occurred in this area, these populations should be characterized by a lower allelic richness than coastal populations. More generally, genetic information indicating extinction-recolonization events at large spatial and temporal scale in species of the Amazonian rainforest is beginning to build up (see the example of the genus *Mauritia*; Rull 1998). Caron et al. (2000) also showed that central populations of *Dycorinia guianensis*, another rainforest tree species from French Guiana, harbor more cpDNA variation than coastal populations. Furthermore, Pleistocene climatic changes have been associated with speciation in the Neotropical genus *Inga* (Richardson et al. 2001). These recent results are consistent with those presented in this study and suggest that rainforest species such as *V. americana* experienced an important contraction of their range and subsequent recolonization during Pleistocene and Holocene climatic change.

The genetic data presented here can even tentatively be used to build colonization scenarios in French Guiana. Populations of *V. americana* from the four most stable areas (Granville 1982; Tardy 1998) all harbor at least one of the chlorotypes observed in recolonized areas (i.e., the coast), suggesting recolonization from these four putative refugia. This is supported by the observed gradient of allelic nDNA richness. However, the recolonization processes were probably more complex than this basic scenario. Historical data indeed provides a picture of population extinction-recolonization in the Neotropics that is more complex than in Europe (e.g., Bush 1994; Dynesius and Jansson 2000; Pennington et al. 2000). Forest recolonization in Europe during the late Pleistocene and Holocene was a steady expansion over large areas, at least after the younger Dryas period (Hewitt 1999). In the Neotropics, the rainforest was affected by several drier periods interspersed with more favorable periods of expansion during the Holocene, leading to several events of dramatic population extinction-recolonization (Charles-Dominique et al. 1998; Rull 1998; Tardy 1998; Mayle et al. 2000). Furthermore, three isolated Pleistocene refugia have been clearly defined in southern Europe (Taberlet et al. 1998; Hewitt 1999). In French Guiana and probably more generally in the Neotropics, the spatial location of favorable areas for rainforest species should have strongly varied throughout the Pleistocene and Holocene periods in relationship with this succession of rainforest contraction-expansion (e.g., Prance 1982; Bush 1994). It is also possible that several small areas, such as gallery forests along rivers, allowed the local maintenance of rainforest species (Oliveira-Filho and Ratter 1995; Aide and Rivera 1998). However, the pattern of allelic richness of *V. americana* in French Guiana suggests that the four refuge areas assumed by Granville (1982) and Tardy (1988) were probably the most important for the maintenance of genetic diversity in this species. Furthermore, *V. americana* does not flourish on flooded soils (Forget et al. 1999), suggesting that large populations of this species cannot be maintained in gallery forests, which therefore played a limited role. Models of population genetics assuming different scenarios of extinction-recolonization (see Austerlitz et al. 1997, 2000; Le Corre and Kremer 1998) should be the next step to more precisely retrace the history of rainforest species.

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